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Cr(VI) reduction into Cr(III) as a mechanism to explain the low sensitivity of *Vibrio fischeri* bioassay to detect chromium pollution

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Abstract

Vibrio fischeri bacteria, used as a biological target in either acute or chronic toxicity tests, display a low sensitivity to Cr(VI). This phenomenon could be due to the capacity of these bacteria to reduce Cr(VI) into Cr(III). This reducing capacity was found to depend on culture medium composition, pH value, incubation time and the presence of a carbon source. It also depends on the nature of the carbon source, glucose being more efficient than glycerol. This is probably related to differences in bacterial metabolism when given either glucose or glycerol. The thermostable Cr(VI)-reducing activity found in the supernatants of *V. fischeri* cultures grown on glucose suggests that, under these conditions, the bacteria release non-proteic reducing substances which have not been identified yet.

Keywords: Chromium toxicity; Cr(VI) reduction; Bacterial reducing capacity

1. Introduction

Hexavalent chromium is widely used in industries such as leather tanning, electroplating or pigment production. Due to high water-solubility and toxicity, Cr(VI)-containing wastes are considered as severe pollutants. Detection of low levels of environmental toxicity is generally supposed to be conveniently achieved by looking at first signs of impact on living organisms. Consequently, several bioassays were proposed for evaluating toxicity of industrial effluents which may contain hazardous metalloids or metals. Growth and metabolic alterations in mammalian cultured cells (Delmas et al., 2000; Gaubin et al., 2000), *Daphnia magna* mobility test (Liu et al., 2002) or duckweed test (Ince et al., 1999) have been used to determine toxicity of metallic ions. The Microtox® bioassay, based on fading of light emitted by *Vibrio fischeri* bacteria (strain NRRLB-11177), is also frequently used for assessing the toxicity of environmental samples. This bioassay, usually carried out at 15 °C for 15 min, has been shown to be very sensitive to a wide range of chemicals and metallic salts (Kaiser and Ribó, 1988; Ribó et al., 1989). Surprisingly, the adverse effect of chromium(VI) when using this test was found to be weak (Villaescusa et al., 1997) as compared with hexavalent chromium toxicity to eukaryotic cells (Delmas et al., 2000). Long-lasting (up to 96 h) exposures of *V. fischeri* to Cr(VI) under the conditions of a viability inhibition test also failed to reveal the expected toxicity (Fulladosa et al., 2005). Besides, in the case of the Microtox test,

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Cr(VI) toxicity was found to further decrease when the pH of the assay medium increased (Villaescusa et al., 1997). Such changes in toxicity as a function of pH, which also occurs with arsenate solutions (Fulladosa et al., 2004), have generally been attributed to changes in speciation of the chemical agents (http://www.kemi.kth.se/medusa). Furthermore, not only chemical speciation but also metal oxidation state can influence metal toxicity. Thus, enzymatic detoxification (generally based on redox reactions) converting toxic elements to less toxic or less bio-available species may be considered as one of the protective mechanisms which can provide a resistance against dangerous metalloids and metals (Silver and Phung, 1996) as it was reported to be the case in bacteria found in the presence of Fe(III), U(VI), Mn(IV) or Tc(VII) (Lovley et al., 1989; Myers and Nealson, 1990; Lloyd and Macaskie, 1996). It is interesting to note, however, that chromium, especially under its reduced form, is a useful trace element in living organisms, including higher animals (Mertz, 1976) and, since it increases cells sensitivity to insulin, chromium was proposed as dietary additive to prevent diabetes (Miranda and Dey, 2004; Underwood, 2006).

Intracellular reduction of chromium(VI) to chromium(III) has been reported to be a possible detoxification mechanism in a wide variety of microorganisms. It was demonstrated in several bacterial species including strains of *Pseudomomas* (Horitsu et al., 1987; Bopp and Erhlich, 1988), *Streptomyces* (Laxman and More, 2002; Desjardin et al., 2003) and *Bacillus* sp (Garbisu et al., 1998). Sulfate-reducing marine bacteria were also found to display a Cr(VI) reduction capacity (Fude et al., 1994; Sisti et al., 1996; Cheung and Gu, 2003). In some cases, the reduction of Cr(VI) was shown to take place in the extracellular domain due to the excretion of metabolites possessing a chemical reducing power. For example, *Thiobacillus ferrooxidans* was shown to generate sulphite and thiosulfate which reduce Cr(VI) at low pH (Sisti et al., 1996); sulfate-reducing bacteria were proven to indirectly reduce Cr(VI) via the production of sulfide (Fude et al., 1994). In other cases, bacteria were found to use carbohydrates like glucose (Bopp and Erhlich, 1988), glycerol (Desjardin et al., 2003) or sucrose (Schmieman et al., 1998) as reducing-carbon and energy sources to achieve the Cr(VI) reduction.

These data, taken together with the fact that Cr(VI) was found to display almost no toxicity on *V. fischeri*, prompted us to study the Cr(VI) reducing capacity of *V. fischeri* in order to explain the resistance of these bacteria to this metal. The effect of different media, different concentrations of carbon sources, namely glucose and glycerol, effect of pH, localization of Cr(VI) reduction phenomenon and effect of cupric ions on the reduction process were investigated. This work is a contribution to the understanding of the complex mechanisms of biological resistance to chemical pollutants. It also calls attention to the different sensitivity of organisms which are used as biological target in bioassays when monitoring environmental pollution.

2. Material and methods

2.1. Bacteria, growth conditions and tested culture media

The marine Gram-negative luminescent bacterium *V. fischeri* (strain NRRLB-11177), obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) was used throughout this study. The lyophilized bacteria were rehydrated with nutritive Sea Water Medium (SWM). This medium was made of artificial sea water (ASW) purchased from Panreac (Barcelona, Spain) (ASW composition: 28.1 g/l NaCl, 0.77 g/l KCl, 1.6 g/l $CaCl_2 \cdot 2H_2O$, 4.8 g/l $MgCl_2 \cdot 6H_2O$, 0.11 g/l $NaHCO_3$, 3.5 g/l $MgSO_4 \cdot 7H_2O$) supplemented with 10 g/l meat extract for bacteriology and 10 g/l bacteriological peptone. SWM was prepared according to the DSMZ instructions: meat extract and bacteriological peptone were dissolved into 250 ml hot water, adjusted to pH 7.8, boiled for 10 min, cooled down and readjusted to pH 7.3. In parallel, 750 ml ASW were prepared and autoclaved at 121 °C for 20 min. When cooled, the two media were combined. Cultures were grown in SWM at 20 °C and aerated by a 130 rpm spinning agitation.

For testing the ability of bacteria to reduce Cr(VI), various media were used, namely: (A) 0.34 M NaCl medium (pH 6.0) in order to be close to the conditions of the Microtox® and viability inhibition assays. (B) M63 medium, commonly used in microbiology (Miller, 1972), has been modified by adding sodium chloride and it is referred as mM63, containing 0.34 M NaCl, 0.1 M KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 3.2 μM $FeSO_4$, 1.6 mM $MgSO_4$ and 3 μM thiamine, adjusted to pH 6.8 and supplemented with either 10 g/l glucose (referred as mM63+Glc) or 3 g/l glycerol (referred as mM63+Glyc). (C) modified Vogel-Bonner medium (McLean and Beveridge, 2001), containing 0.34 M NaCl, 60 mM K_2HPO_4, 20 mM Na_2HPO_4, 15 mM NH_4Cl, 1 mM $MgSO_4$, 2 g/l citric acid (pH 6.0) and supplemented with 7.5 g/l glucose (referred as mVB). (D) SWM (described above) was also tested. All media were autoclaved at 121 °C for 20 min. In all cases, glucose and glycerol solutions were autoclaved separately.

2.2. Chromium analysis

Cr(VI) was analyzed by a colorimetric method using the diphenylcarbazide (DPCZ) reaction (Desjardin et al., 2003). Fifty microliter of DPCZ solution (0.3 g of chemically pure diphenylcarbazide diluted to a final volume of 500 mL by adding 100 mL 95% ethanol and 400 mL of 3.6 N sulphuric acid) was added to 1 ml of appropriately diluted sample. DPCZ reacts with chromate forming a purple complex with a maximum absorbance at 540 nm. Samples were periodically collected under sterile conditions and, when necessary, centrifuged before dilution to remove suspended cells and avoid turbidity. Absorbance measurements were performed with a Spectronic 401 spectrophotometer (Milton Roy, France).